The Effect of Trimethoprim/Sulfamethoxazole on Friend Erythroleukemia Cells

By S. E. Steinberg, C. L. Campbell, P. S. Rabinovitch, and R. S. Hillman

Cultures of Friend erythroleukemia cells were subjected to the antibiotics trimethoprim (T) and sulfamethoxazole (S) at levels equal to or below the usual therapeutic range. At T 8 μg/ml and S 40 μg/ml, cell growth was arrested, cells appeared megaloblastic, and the examination of cell-cycle distribution by flow microfluorimetry revealed arrest in S phase. With a tenfold reduction in drug levels (T, 0.8 μg/ml; S, 4 μg/ml) cell growth was less markedly inhibited, morphology remained megaloblastic, and S-phase block was still dramatic. A further tenfold reduction (T, 0.08 μg/ml; S, 0.4 μg/ml), well below effective antibacterial levels, allowed normal cell growth and morphology but DNA synthesis was still inhibited. Additions of folinic acid at 100 ng/ml averted all drug effects. Thus, T/S can affect cell replication even at levels well below those usually employed and could prolong the rate of recovery of hematopoietic cells in the myelosuppressed patient.

The combination of trimethoprim (T) and sulfamethoxazole (S) was introduced in the 1960s for the treatment and prophylaxis of bacterial infections. Recently, it has been used in the treatment of Pneumocystis carinii infections, as well as for prophylaxis in the neutropenic patient.

The effectiveness of the trimethoprim/sulfamethoxazole combination stems from the ability of each drug to inhibit a different step in folate metabolism. Sulfonamide competitively inhibits the incorporation of p-aminobenzoic acid into the pteridine ring and impair the growth of organisms that are unable to utilize preformed folates but must synthesize them from p-aminobenzoic acid.

Trimethoprim binds dihydrofolate reductase in a manner analogous to methotrexate. In doing so, it inhibits the regeneration of 5, 10-methylene tetrahydrofolate, which is a methyl group donor in the formation of dTMP, one constituent of DNA. Species variation in drug-reductase binding determines its therapeutic efficacy. The dihydrofolate reductase of the protozoa, P. berghei, is 4300 times more sensitive (K_i = 1.1 x 10^{-7} M) to trimethoprim than the corresponding human hepatic enzyme (K_i = 3.4 x 10^{-4} M), while the E. coli enzyme is 60,000 times more sensitive (K_i = 5 x 10^{-9} M).

Even the usual doses of trimethoprim and sulfamethoxazole have been associated with hematologic abnormalities in otherwise normal individuals, and higher doses are now commonly used in patients with compromised marrow function. Therefore, these studies were designed to investigate in more detail the effects of varying doses of the drugs on hematopoietic cells.

MATERIALS AND METHODS

A long-term culture of Friend murine leukemia cells (line M745, obtained originally from Dr. David Housman, Boston, Mass.) was maintained free of contamination with mycoplasma in suspension in alpha-modified Eagle's media (Gibco F19) with 10% fetal calf serum, 1% penicillin and streptomycin, in a humid 5% CO_2 atmosphere at 37°C. The media contained 1000 ng/ml of folic acid (pteroylglutamic acid), as measured by the aseptic method. Trimethoprim (16 mg/ml) in combination with sulfamethoxazole (80 mg/ml) was obtained as Bactrim in 3-ml vials from Roche Pharmaceuticals, N.J. Folic acid was obtained as leucovorin calcium, 3 mg/ml, from Lederle Laboratories, Division American Cyanamid Company, Pearl River, N.Y.

For purpose of the experiment, cells were transferred while in log phase growth from the stock media into test media identical except that they contained only 100 ng/ml of folic acid plus varying concentrations of trimethoprim/sulfamethoxazole and folinic acid (5-formyl tetrahydrofolate, leucovorin). A folate level of 100 ng/ml was chosen because it allows normal growth and morphology without providing a large folate excess. Cultures were initially plated at 100,000 cells/ml in a total of 10 ml of media. Final drug concentrations were selected to duplicate clinical levels and prepared by serial dilution with media and confirmed by measurements of drug levels.

The highest dose level, T, 8 μg/ml (2.8 x 10^{-3} mM) and S, 40 μg/ml (1.6 x 10^{-2} mM), is equivalent to that usually attained in the treatment of Pneumocystis carinii. The moderate dose level, T, 0.8 μg/ml (2.8 x 10^{-4} mM) and S, 4 μg/ml (1.6 x 10^{-3} mM), is at or below that usually recommended as prophylaxis against Pneumocystis carinii in immunosuppressed patients and bacterial infections in neutropenic patients, or for treatment of routine bacterial infections. The lowest dose level, T, 0.08 μg/ml (2.8 x 10^{-5} mM) and S, 0.4 μg/ml (1.6 x 10^{-3} mM), is well below that usually considered of value therapeutically. Finally, to determine the amount of folinic acid required to avert drug effects, folinic acid was included in cultures at the high-dose drug levels in concentrations of 10, 100, and 1000 ng/ml, prepared by serial dilution with media, and confirmed by the aseptic L. casei method.

Several parameters of the test group were examined. Growth characteristics were determined by daily cell counts utilizing trypan blue exclusion to measure viability. Morphological examination was...
performed daily on Wright-stained preparations. The mean corpuscular volume (MCV) was measured on a Model S Coulter Counter at 72 hr. DNA analysis was performed by flow microfluorometry of cells in log phase growth. Techniques and instrumentation (ICP-11, PHYWE Co., Gottingen, Germany) have previously been described. Briefly, cells fixed in 70% ethanol were stained for 10 min each with ethidium bromide, 25 μg/ml (Sigma Chemical Company, St. Louis, Mo.) and with mithromycin, 50 μg/ml (Pfizer Laboratories, New York, N.Y.) containing 7.5 mM of MgCl₂ in 12.5% ethanol. Fluorescence pulse-height distributions (DNA content histograms) of 10⁴–10⁶ cells were accumulated in a Tracor-Northern NX-600 series multichannel analyzer and loaded, via an RS-232 port, into a PDP-11/03 minicomputer. A set of Fortran-coded programs permit data storage on floppy disks as named files, as well as properly labeled display on a Tektronix graphics terminal. Figures shown in this text are direct photographs of such graphic displays. Quantitative evaluation of DNA histograms (percentage of cells with DNA content equivalents to G₁, S, G₂, and M) was performed using curve-fitting algorithms described by Dean and Jett.

Statistical analysis was performed by the one-tailed Student's t test.

RESULTS

Growth Characteristics (Fig. 1)

Control experiment cells grew in log phase between 24 and 48 hr until a plateau was reached at approximately 2–2.5 × 10⁶ cells/ml. At the highest test dose, cell growth was completely inhibited, while at the intermediate dose, growth was improved but did not reach control levels. At the low dose, cell growth was equivalent to the control.

Morphology

When compared to controls, cells grown in high and moderate drug levels demonstrated markedly megaloblastic features at 72 hr. There were many very large cells with dyspoietic nuclei, finely distributed chromatin, and indistinct nuclear membranes. Cells grown at low drug concentrations appeared similar to controls.

Mean Corpuscular Volume

The MCV of control cells was 98 ± 2.3. At 72 hr, the MCV of cells cultured in high (122 ± 4.3) or moderate (115 ± 3.1) drug levels were significantly larger than controls (p < 0.01), whereas the MCV of cells cultured in low doses (102 ± 4.1) was similar to controls.

DNA Analysis (Fig. 2)

The DNA histogram of control cells is shown in Fig. 2A. Computer analysis revealed 17% of the cells had a 2N complement of DNA corresponding to the G₁ phase, 68% contained more than 2N but less than 4N amount of DNA and therefore were in the synthetic (S) phase, and the remaining 15% contained a full 4N complement of DNA corresponding to the G₂ or mitotic (M) phase (Table 1). DNA distribution of

![Fig. 1. Growth curves of cells exposed to various drug concentrations: control (A); T, 8 and S, 40 μg/ml (B); T, 0.8 and S, 4 μg/ml (C); T, 0.08 and S, 0.4 μg/ml (D).](image)

![Fig. 2. Fluorescence pulse-height histograms (DNA content distributions) of 10⁴–10⁶ cells exposed to various drug concentrations: control (A); T, 8 and S, 40 μg/ml (B); T, 0.8 and S, 4 μg/ml (C); T, 0.08 and S, 0.4 μg/ml (D).](image)
Table 1. Cell-Cycle Distribution at Varying Drug Doses

<table>
<thead>
<tr>
<th></th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17</td>
<td>68</td>
<td>15</td>
</tr>
<tr>
<td>T, 8 S, 40 µg/ml</td>
<td>5</td>
<td>83</td>
<td>12</td>
</tr>
<tr>
<td>T, 0.8 S, 4 µg/ml</td>
<td>7</td>
<td>83</td>
<td>10</td>
</tr>
<tr>
<td>T, 0.08 S, 0.4 µg/ml</td>
<td>13</td>
<td>74</td>
<td>13</td>
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cells grown at the high drug dose (Fig. 2B, Table 1) showed a marked accumulation of cells in the S phase, suggesting a defect in the synthesis of the full 4N complement of DNA necessary for mitosis. Moderate dose cells (Fig. 2C, Table 1) showed the same marked block in the S phase of cell cycle. Although the growth curve, morphology, and MCV of low dose cells were similar to controls, examination of the DNA histogram showed inhibition of the synthetic phase of the cell cycle, though not to the same degree as at higher doses (Fig. 2D, Table 1).

**Effect of Folinic Acid**

At 10 ng of folinic acid, cell growth at the high dose concentration was somewhat enhanced, whereas at 100, 500, and 1000 ng/ml, growth was restored to nondrug control levels (Fig. 3). Furthermore, at 1000 ng/ml, all abnormalities, including those of the DNA synthesis, reverted to normal.

**DISCUSSION**

The use of trimethoprim/sulfamethoxazole has increased as a result of proven effectiveness in the prophylaxis and treatment of *Pneumocystis carinii* infections and prophylaxis in chemotherapy-related neutropenia.²⁻⁴ Ease of administration, low cost, and a low incidence of side effects have also contributed to its popularity.⁴ Because trimethoprim is known to affect folate-dependent DNA synthesis,¹² the present in vitro studies were designed to examine drug dosage effects on hematopoietic cells as well as the level of folinic acid necessary to avert them. Since the effect on DNA synthesis is more likely to be evident in a rapidly proliferating population, Friend erythroleukemia cells were considered ideal for such studies.

At both high and moderate drug levels, in vitro cell growth was markedly inhibited. Even at subtherapeutic doses, when growth and morphology were normal, abnormal DNA synthesis was detected by flow microfluorometry. As might be expected, all drug effects were averted by the inclusion of 100 ng/ml or more of folinic acid in the media. An equivalent level in humans is achieved with daily doses of approximately 15 mg of folinic acid.¹³ At the same time, supplementation of folate in vivo does not interfere with the therapeutic effectiveness of the drug, since virtually all bacteria lack the ability to utilize exogenous folate.¹⁴

While clinical defects in hematopoiesis have been reported with trimethoprim/sulfamethoxazole,⁶ they have not been as common or as severe as these in vitro data might predict. There are several possible explanations for this. First, most clinical surveys of drug effect have studied patients with normal nutritional status,³ while our in vitro experiments examined a cell population grown with marginal folate supply. Although prolonged trimethoprim/sulfamethoxazole administration does not affect serum folate levels,¹⁵,¹⁶ studies have shown that patients with nutritionally impaired folate metabolism (e.g., folate or B₁₂ deficiency) are at increased risk for drug toxicity.¹⁷,¹⁸ Second, a greater availability of reduced folates in vivo might be expected in as much as they are more readily transported into the cell and either bind dihydrofolate reductase more avidly (dihydrofolate), or bypass the trimethoprim/sulfamethoxazole block (methyltetrahydrofolate). In either event, drug effect would be reduced.¹⁹,²² Third, prolonged exposure to trimethoprim/sulfamethoxazole or other dihydrofolate reduc-
tase inhibitors (methotrexate) in vivo can increase dihydrofolate reductase levels. Such enzyme induction would tend to protect against drug effects. In addition, the level of cell proliferation may be an important factor. Surveys of drug toxicity have been carried out on patients with relatively normal counts and basal marrow function, whereas a highly proliferative bone marrow recovering from intensive chemotherapy may well be more sensitive. This would be consistent with the observation that patients with stimulated marrows are very sensitive to the limitation of folate supply. Last, toxicity in past studies has been defined by abnormalities of cell counts, indices, and smears, all of which are relatively insensitive indicators of disordered folate metabolism when compared to DNA analysis by flow microfluorometry.

In view of these considerations, assessment of the clinical implications of this study will require more precise measurements of the in vivo effects of trimethoprim/sulfamethoxazole on patients with proliferative marrows. The in vitro data are not sufficient to suggest that prophylactic use of trimethoprim/sulfamethoxazole in patients undergoing chemotherapy should be curtailed. Rather, when such a patient has an unexpectedly prolonged period of cytopenia, the possibility of drug-related impairment of cell replication should be considered, and treatment with folinic acid implemented.

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REFERENCES

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